

CONCISE COMMUNICATION

The White Morphotype of *Mycobacterium avium-intracellulare* Is Common in Infected Humans and Virulent in Infection Models

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Isolates of *Mycobacterium avium-intracellulare* (MAI) form multiple colony types named red-opaque, white-opaque, red-transparent (RT), and white-transparent (WT). The newly discovered WT morphotype is multidrug resistant relative to other variants *in vitro*. To determine whether the WT morphotype occurs in humans, 32 MAI-positive clinical samples from 2 sites were plated directly onto indicator agar without prior passage *in vitro*. WT was the predominant morphotype in 26 (81%) of these samples and was absent in only 2 samples. WT variants grew better than isogenic RT variants in mouse and human macrophage models of infection, and RT clones that passed through such systems underwent rapid shifts to the WT morphotype. The RT morphotype was heterogeneous with regard to infectivity. In summary, the white morphotype was common in humans and was favored in disease models. It may play an important role in the establishment and persistence of MAI infection.

Isolates of the *Mycobacterium avium-intracellulare* (MAI) complex commonly segregate into smooth-transparent, smooth-opaque, and rough colony-type variants. Transparent variants predominate in patient samples, grow better in macrophage and animal models, and are more drug resistant than other morphotypes. Opaque variants predominate after passage *in vitro* but are considered to be attenuated [1–4]. Rough variants also appear after passage *in vitro*.

We recently described a new morphotypic switch in MAI, termed red-white, that is independent of the opaque-transparent and smooth-rough switches [5, 6]. The red-white variation becomes visible when opaque colonies are grown on agar medium that contains the lipoprotein stain Congo red (CR). White-opaque (WO) variants are more resistant to multiple antibiotics *in vitro* than are isogenic red-opaque (RO) variants [5].

Transparent derivatives of RO and WO clones, termed red-transparent (RT) and white-transparent (WT), are distinguishable in a quantitative CR binding assay but not by visual inspection [6]. Red-to-white switching has not been observed *in vitro* in the absence of selection, but white-to-red switching is more common. Certain reference strains, including the source of DNA for the *M. avium* genome sequence project (strain 104), are stable red variants that do not form white colonies [6]. Among clinical isolates that form both morphotypes, red and white variants generally do not differ with regard to growth on laboratory media. However, WO and WT variants are considerably more resistant to multiple antibiotics (clarithromycin, ciprofloxacin, and rifamycin drugs) than are their isogenic RO and RT counterparts *in vitro* [5, 6]. Susceptibility to these drugs *in vivo* remains to be determined.

Many bacterial species form multiple colony types, only a subset of which is important in human infection. A morphotype that exhibits an interesting phenotype, such as WT's multidrug resistance *in vitro*, is relevant to human disease only if it is virulent and occurs in infected humans. To test the significance of the WT morphotype, we plated clinical isolates of MAI directly onto CR agar without prior passage *in vitro*, to quantify the occurrence of the white morphotype. We also assessed growth and morphotypic selection in nonhuman models of infection.

Materials and Methods

Bacterial strains and culture media. Bacteria were grown on Middlebrook-albumin-glycerol (MAG)–CR agar plates [5] or on Middlebrook 7H9 broth with albumin-dextrose-catalase enrichment. The derivation of *M. avium* clones used for mouse and

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macrophage infections has been reported elsewhere [5, 6]. In brief, laboratory stocks of clinical isolates HMC02 and HMC10 were plated onto CR agar to obtain readily discernible RO and WO clones, named HMC02-RO, HMC02-WO, HMC10-RO, and HMC10-WO. RT and WT clones, which are not easily distinguishable by visual inspection but differ in a quantitative CR binding assay, were derived in vitro from these RO and WO clones [6]. All clones of HMC02 were identical by IS1245 mapping, as were all clones of HMC10 [5].

Collection and analysis of clinical samples. Thirty-six primary MAI cultures were obtained from Carolyn Wallis (Harborview Medical Center, Seattle) and Marjorie Beggs (J. L. McClellan Memorial Veterans Hospital, Little Rock, AR). Cultures had been grown from decontaminated patient samples in Bactec bottles or Mycobacterium Growth Indicator Tubes (Becton-Dickinson) or Lowenstein-Jensen slants. All had been identified as MAI by Accuprobe testing (Gen-Probe). Only 1 culture per patient was delivered, and all patient identifiers were removed before delivery. On receipt, samples were assigned arbitrary numbers, were streaked onto MAG-CR, and were incubated at 37°C. Four of the 36 cultures were contaminated. The remaining 32 samples formed transparent colonies typical of primary MAI patient isolates [7–9]. After 4 weeks of incubation, transparent colonies differentiated to the opaque morphotype, which allowed us to determine whether they were red or white. Isolates were divided into 4 categories: W (all colonies were white); W > R (more white colonies than red colonies); R > W (more red colonies than white colonies); and R (all colonies were red).

The species (*M. avium* or *M. intracellulare*) of each primary clinical isolate was determined by the rDNA-targeted polymerase chain reaction (PCR) approach of Chen et al. [10]. Cells were removed from MAG-CR plates by running loops over broad swaths of growth, such that all visible colony types were picked. DNA was extracted by bead beating, and separate PCR amplification reactions were performed by use of the MAV primer set, specific for the 16S rRNA gene of *M. avium*, and the MIN primer set, specific for the 16S rRNA gene of *M. intracellulare* [10].

Simulated decontamination. RT or WT colonies picked from 2-week-old MAG-CR plates were suspended in sterile deionized water to $\sim 2 \times 10^6$ cfu/mL. Two milliliters of each suspension was transferred to polypropylene centrifuge tubes that contained equal volumes of N-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) reagent (2% NaOH, 1.48% trisodium citrate, and 0.5 mg/mL NALC). The mixtures were incubated at room temperature for 15 min and then were neutralized by the addition of 6 mL of phosphate buffer (60 mM Na₂HPO₄ and KH₂PO₄ [pH 6.8]). Tubes were centrifuged for 15 min at 5000 g, and pellets were resuspended in 1 mL of sterile water. Suspensions then were serially diluted 10–10,000-fold in sterile water; 100 μ L of each dilution was plated for viable counting.

Disease models. Infection of C57BL/6 mice was carried out as described elsewhere [11]. In brief, 6–8-week-old female mice (Jackson Laboratories) were infected intravenously with 3×10^7 cfu of either WT or RT clones. Inocula were plated for quantification and to confirm morphotypic purity. Groups of infected mice (10 per group) were killed at weeks 2, 4, and 6. At each time point, the spleen and liver were aseptically removed, weighed, and then homogenized in 5 mL of Middlebrook 7H9 broth, using a tissue

homogenizer. Tissue suspensions were serially diluted in 7H9 broth and were plated. Colonies were counted after 21 days and were reported as colony-forming units per gram of organ tissue. To determine the CR staining phenotype of cells grown in mice, suspensions were plated onto MAG-CR and were incubated at 37°C for 21–28 days, during which time colonies became opaque. CR staining phenotypes of the opaque colonies were recorded.

Human mononuclear phagocytes were obtained from blood bank white cell concentrates and were infected with MAI, as described elsewhere [6, 12, 13]. To quantify intracellular colony-forming units, macrophage lysates were serially diluted, plated, and counted after 10 days at 37°C. CR staining phenotypes were determined as with mouse-grown cells.

Results

The white morphotype is common in infected humans. To assess the morphotypic composition of MAI in clinical samples, we plated the contents of primary diagnostic cultures directly onto MAG-CR agar. In contrast to the procedures of an earlier study [5], these cultures had never been transferred in vitro. MAI-positive cultures were obtained from clinical laboratories in 2 US cities. Bacteria in these samples formed transparent colonies that switched to opaque after extended incubation, which allowed us to observe CR staining (table 1). White was the predominant morphotype (W or W > R) among colonies formed by 26 (81%) of these samples. White colonies were present but were outnumbered by red colonies (R > W) in 4 (13%) samples and were absent (R) in 2 (6%) samples.

These clinical isolates had been identified as MAI by commercial gene probes, but their species (*M. avium* or *M. intracellulare*) had not been determined by the clinical laboratories. To determine whether CR staining differed between species, we speciated the 32 isolates by rDNA analysis [10]. All but 2 of the 32 isolates were assigned to either *M. avium* or *M. intracellulare* by this method (table 1). The 2 exceptions, AMVH15 and HMC44, were provisionally assigned to the MAC_x category. White was the most frequently observed morphotype, regardless of species, geographical site, or the composition of the commercial primary culture medium. These results show that the white morphotype is common, and possibly predominant, in infected humans.

Most specimens submitted to clinical laboratories for mycobacterial culture are decontaminated by exposure to 1% NaOH, which selects against competing rapidly growing microorganisms [14]. Because white variants are resistant to some antimicrobial agents in vitro, we had to consider the possibility that the predominance of the white morphotype in clinical laboratory samples may have been due to the selective pressure of NaOH processing. To test this possibility, isolated RT and WT variants of 2 clinical isolates were processed according to a standard NALC-NaOH protocol. In 5 repetitions of this experiment conducted in triplicate on strain HMC02 and 3 repetitions conducted in triplicate on strain HMC10, there was no significant difference in the survival of RT and WT clones. This suggests that specimen processing is insufficient to account for the

high prevalence of white variants among our clinical isolates. Another possible selective step, growth in primary cultivation medium, was also unlikely to have played a role, because we have not observed consistent differences in growth rates between red and white variants on Middlebrook or Lowenstein-Jensen media [5, 6]. So far, the only conditions found to be selective for the white morphotype are intracellular growth in mice and growth in human macrophages, as described below. Therefore, the predominance of the white morphotype shown in table 1 was most likely due to selection in host tissues.

Table 1. Species and red-white morphotypes of clinical isolates.

Isolate	Primary culture medium	Site	16S rDNA PCR primer set		Congo red morphotype
			MAV	MIN	
AMVH1	Bactec	Little Rock	—	+	W>R
AMVH2	Bactec	Little Rock	—	+	R>W
AMVH3	Bactec	Little Rock	—	+	W>R
AMVH4	Bactec	Little Rock	—	+	W
AMVH5	Bactec	Little Rock	—	+	W
AMVH6	Bactec	Little Rock	—	+	W ^a
AMVH7	Bactec	Little Rock	+	—	W
AMVH8	Bactec	Little Rock	—	+	W
AMVH9	L-J slant	Little Rock	—	+	W>R
AMVH10	L-J slant	Little Rock	—	+	W>R
AMVH11	L-J slant	Little Rock	—	+	W
AMVH12	L-J slant	Little Rock	—	+	W>R
AMVH13	L-J slant	Little Rock	—	+	W
AMVH14	L-J slant	Little Rock	+	—	W>R
AMVH15	L-J slant	Little Rock	+	+	R>W
HMC31	MGIT	Seattle	+	—	W ^a
HMC32	MGIT	Seattle	+	—	R>W
HMC33	MGIT	Seattle	+	—	R>W ^a
HMC34	MGIT	Seattle	+	—	R
HMC35	MGIT	Seattle	—	+	W>R
HMC36	MGIT	Seattle	+	—	W>R
HMC37	MGIT	Seattle	+	—	W>R
HMC38	MGIT	Seattle	—	+	W>R
HMC39	MGIT	Seattle	+	—	W>R
HMC40	MGIT	Seattle	—	+	W>R
HMC41	MGIT	Seattle	+	—	W
HMC42	MGIT	Seattle	+	—	W ^a
HMC43	MGIT	Seattle	—	+	W
HMC44	MGIT	Seattle	—	—	W
HMC45	MGIT	Seattle	+	—	R ^a
HMC46	MGIT	Seattle	+	—	W
HMC47	MGIT	Seattle	+	—	W
13950 ^b	NA	NA	—	+	W
35717 ^b	NA	NA	+	—	R ^a
104 ^b	NA	NA	+	—	R

NOTE. L-J, Lowenstein-Jensen; MAV, *Mycobacterium avium*-specific primer set; MGIT, Mycobacterium Growth Indicator Tube; MIN, *M. intracellulare*-specific primer set; minus sign (—), negative by polymerase chain reaction (PCR); NA, not applicable; plus sign (+), positive by PCR; R, red; R>W, mixed (more red than white); W, white; W>R, mixed (more white than red).

^a Pink colonies also were present. Such colonies have been described elsewhere [5, 6] but have not been characterized.

^b These laboratory reference strains were included as PCR controls.

Growth of the white morphotype in disease models. When human monocyte-derived macrophages were infected with RO, WO, RT, and WT variants of 2 clinical isolates, HMC02 and HMC10, the WT variants survived or increased in number, whereas the other morphotypes were killed [6]. To further test the virulence of WT variants in relation to isogenic RT variants, we infected C57 BL/6J mice intravenously with RT and WT clones of clinical isolates HMC02 and HMC10. We also infected mice with strain 104, the source of DNA for the *M. avium* genome sequence project at The Institute for Genomic Research (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>). Strain 104 is a stable RT clone that does not form white colonies [6]. Inocula ranged in size from 1.6×10^7 to 7.5×10^7 cfu per mouse. Spleen and liver samples taken 2, 4, and 6 weeks after infection were plated to determine colony count. Viable counts of WT clones HMC02-WT3 and HMC10-WT2 and of the stable RT strain 104 increased 2–10-fold between weeks 2 and 6 after infection, consistent with the property of virulence (figure 1). The RT clone HMC02-RT3 successfully colonized spleens and livers but grew more slowly than its WT counterpart (figure 1, *top*). In contrast, only small numbers of clone HMC10-RT2 were detected in spleens and livers by week 2 (figure 1, *center*; note Y-axis scale). This very low level of infectivity is consistent with that found in an earlier study, in which this clone was found to be highly susceptible to killing by human macrophages [6]. Although initially few in number, this clone subsequently grew in both organs, which possibly reflects a morphotypic subpopulation that was more virulent than the predominant HMC10-RT2 population.

Within the 2 isolates that form both morphotypes (HMC02 and HMC10), white variants were favored over red variants in mice. To further test the hypothesis that the white morphotype is favored in vivo, we examined CR-staining morphotypes of colonies formed by bacteria that had been passed through mice and human macrophages. As expected [2], all such colonies were initially transparent and therefore were difficult to characterize by CR staining. However, after extended incubation on MAG-CR, the colonies switched to the opaque morphotype, and CR staining properties became visible. All colonies formed by bacteria obtained from mice or formed by macrophages infected with pure cultures of HMC02-WT3 or HMC10-WT2 cells were white. In contrast, colonies grown from bacteria obtained from mice or grown from macrophages infected with pure cultures of HMC02-RT3 cells were mixed, with red and white colonies appearing in nearly equal numbers (mean \pm SD, $55.9\% \pm 11.1\%$ white). The shift to the white morphotype occurred early (before week 2 in mice), and the percentage of white colonies did not increase with subsequent incubation in mice.

When HMC10-RT2 cells were passed through mice and human macrophages, all colonies came up white. The rapidity of the shift was especially evident in the macrophage model. Colonies grown from macrophages infected with HMC10-RT2 were 100% red when plated 1 day after infection and then

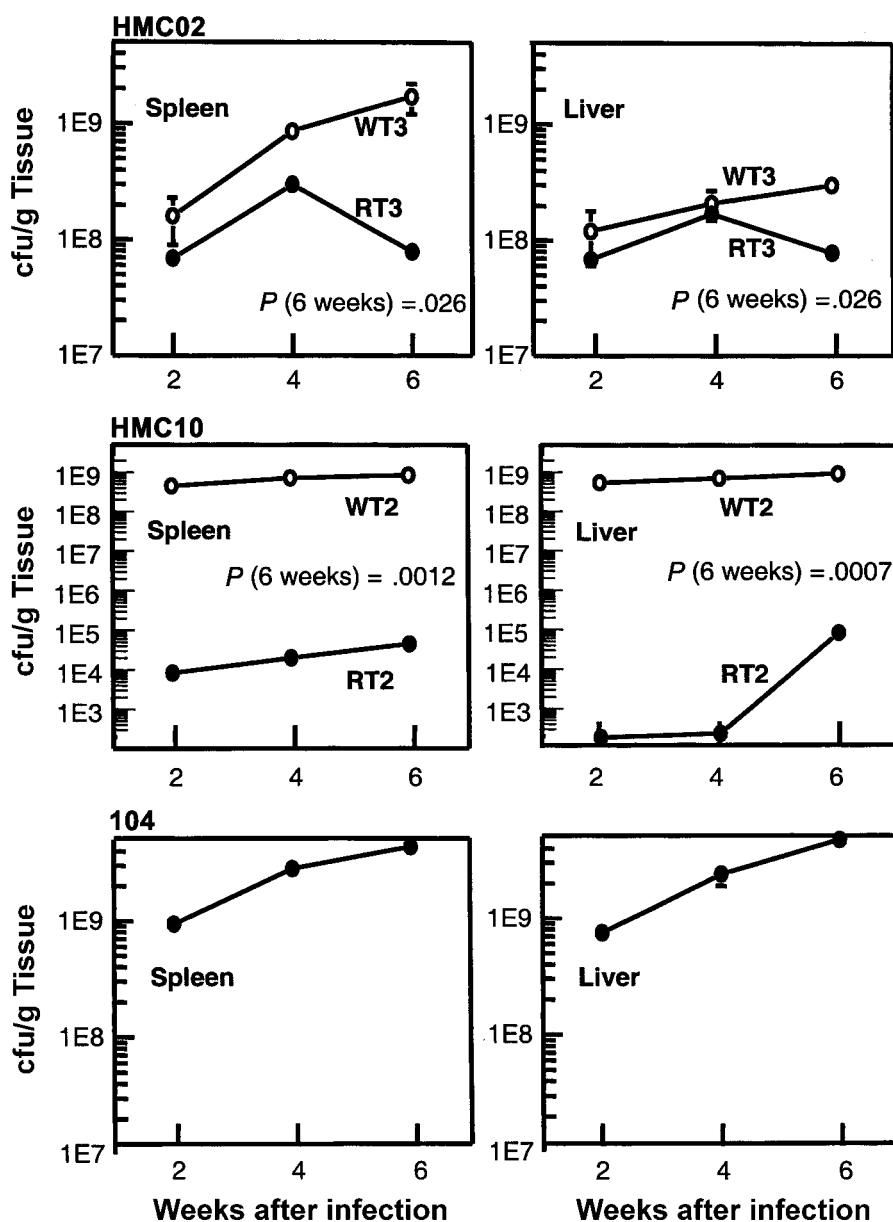


Figure 1. Colonization of mouse liver and spleen tissues by isolated red-transparent (RT) and white-transparent (WT) variants. Mice were infected intravenously with isolated RT (●) or WT (○) variants. *Top*, HMC02-RT3 and HMC02-WT3; *middle*, HMC10-RT2 and HMC10-WT2; *bottom*, strain 104, which forms RT colonies only. At the time of inoculation, samples of inocula were plated for viable counting. Inoculum sizes were as follows: HMC02-RT3, 3.3×10^7 cfu; HMC02-WT3, 3.1×10^7 cfu; HMC10-RT2, 3.2×10^7 cfu; HMC10-WT2, 7.5×10^7 cfu; and 104, 1.6×10^7 cfu. At 3 time points after infection, groups of mice were killed, and colony-forming units in livers and spleens were counted, as described in Materials and Methods. Each data point is the mean and SD of colony-forming units from 10 mice.

were 100% white on days 2, 3, and 4. The rapidity of this shift suggests a true switch, as opposed to selection of a preexisting white subpopulation. Cell numbers did not increase after the red-to-white switch. However, when day 4 colonies grown from HMC10-RT2-infected macrophages were used to infect new macrophages, they grew as well as did clone HMC10-WT2 [6], which indicates that they were fully virulent.

The pronounced red-to-white switch that occurred in mice and macrophages contrasted with growth on culture media, where such a shift has never been observed in strains HMC02 and HMC10 [6]. These observations support the hypothesis that the white morphotype is favored during intracellular growth of these isolates. White colonies isolated from mice and macrophages infected with RT cells were identical in appearance

to colonies of stable white clones, such as HMC02-WT3 or HMC10-WT2. However, they switched back to the red morphotype after only 1 or 2 transfers in vitro. Although morphotypically identical to the stable white clones, they may have been products of less stable mutational or phase variation events.

The ability of WT variants of strains HMC02 and HMC10 to grow in mice and macrophages is consistent with the common occurrence of WT variants in human samples. Bacteria grown in the nonhuman infection models were not subjected to alkaline decontamination or grown in broth media before plating on MAG-CR. This is consistent with the conclusion that the isolation of white variants from host environments is not an artifact of specimen processing.

The red morphotype appears to be relatively heterogeneous with regard to virulence, and there may, in fact, be multiple red morphotypes. RT variants of HMC02 and HMC10 were both less virulent than their isogenic WT counterparts, but to differing degrees. Strain 104 was fully virulent, despite its inability to form white colonies in vitro, and colonies grown from cells of mice infected with pure cultures of strain 104 were 100% red. Consistent with the observation that some red variants can cause disease, 2 of the clinical samples listed in table 1, HMC34 and HMC45, formed no white colonies.

Discussion

We recently observed that white variants are more resistant in vitro than isogenic red variants to multiple antimycobacterial drugs [5, 6]. Experiments are under way to determine whether the white morphotype is multidrug resistant in the murine treatment model. The purpose of the present study was to test the hypothesis that white variants are involved in MAI disease. We found that white variants were common, and possibly even predominant, in primary cultures of MAI that had come directly from clinical laboratories without passage in vitro. Moreover, white variants of 2 clinical isolates were favored in nonhuman disease models. Together, these observations constitute strong evidence that the white morphotype is active in infection.

The apparent selective advantage that white variants have in vivo has not yet been identified. The red-to-white switch occurred early in infection of mice and human macrophages, but the switch did not appear to increase the bacterial growth rate in host environments. Therefore, the white morphotype may inhibit an early step in the host response to infection. When the infecting population is predominantly red, early host responses may be able to control subsequent growth of the bacteria, even after they have switched to the white morphotype.

Red and white phenotypes on CR agar could both occur through >1 mechanism. Therefore, the red and white morphotypes may each be a collection of multiple subtypes. The data reported here suggest that at least some white variants contribute to the progression and persistence of MAI disease in humans. Strain 104 and clinical isolates HMC34 and HMC45 may be

somewhat unusual, in that they do not form white colonies, even when taken from mammalian hosts, but are nonetheless virulent. With the near completion of its genome sequence, strain 104 is likely to become one of the best-characterized MAI strains. However, if we are to gain a complete understanding of how MAI infects susceptible hosts and resists treatment, the white morphotype must also be characterized.

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